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The Effect of pH and Temperature on the Stability and Enzymatic Activity of Prostatic Acid Phosphatase

Studies on the Optimization of a Continuous Monitored Determination of Acid Phosphatase, II.

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Summary: The catalytic activity and the stability of prostatic acid phosphatase were studied with respect to pH and temperature:

1. Enzymatic activity in serum decreases with time, and the rate of decrease depends on pH and temperature. Half life times were estimated.
2. To preserve at least 90% of its original activity, serum must be cooled as soon as possible below room temperature and/or the pH must be lowered to 6.
3. Considering the effect of pH on side reactions and kinetic parameters, a pH of 5.2 is recommended for the assay.
4. Between 25 and 37 °C, the value for $K_{m\,app}$, in the absence of alcohols, is constant within the limits of error. In the presence of alcohols there is a significant increase of $K_{m\,app}$ at lower temperatures, and higher substrate concentrations are needed to avoid nonsaturation of the enzyme. v_{max} increases with temperature. Inactivation is observed above 45 °C, especially in the presence of alcohols.
5. The *Arrhenius* plot shows a strict linear regression between 20 °C and 40 °C, in the presence or absence of 1,4-butanediol, 1,5-pentanediol or 1,6-hexanediol.
6. Temperature conversion factors for catalytic activity were calculated to be: 1.33 (25 to 30 °C), 1.96 (25 to 37 °C) and 1.47 (30 to 37 °C).

Introduction

Proportionality between quantity of enzyme and catalytic activity is an important prerequisite for the determination of catalytic activity as an aid to clinical diagnosis. Temperature is one of the important factors influencing catalytic activity and has subsequently to be controlled. Acid phosphatases (EC 3.1.3.2) may derive in humans not only from different organs but even from different compartments within a given cell e. g. from lysosomes or secretory granules.

In the case of secretory acid phosphatases of the prostate it has been shown that at least 15 different isoenzymes can be separated (1). So far, the physiological substrates of these phosphatases are still unknown. Thus, determination of acid phosphatase depends on artificial substrates, e. g. *p*-nitrophenyl phosphate or naphthyl phosphate, the latter being used in a continuous method developed by *Hillmann* (2) with continuous monitoring. In an attempt to optimize this method our paper deals with temperature dependence of the enzymatic reaction and with the stability of the enzyme in serum.

[1]) Teil der Dissertation (D 26)

With respect to clinical relevance and practicability, immunologic tests (RIA as well as ELISA), even with monoclonal antibodies, are not superior to enzymatic tests (3, 4). In addition, the cost and time for the immunologic determination of acid phosphatase exceed those for the measurement of enzymatic activity. Thus, optimization and standardization of the enzymatic determination of acid phosphatase is still an obligation.

Experimental

Reagents

Fast red TR salt (Sigma F 1500); 1-naphthyl phosphate p. a. (Serva 30130); bovine serum albumin, lyophil. (Serva 11930 control E; other control batches did not match the requirements of the test); 1,4-butanediol (Merck 801532); 1,6-hexanediol (Merck 804308); 1,5-pentanediol (Fluka 76890); sodium acetate (Merck 6267). All other reagents were of the best quality available. Freshly clotted blood was centrifuged, immediately acidified by addition of 2 mol/l acetic acid (20 ml/l) and stored at 4 °C for routine use. In all other experiments pH was corrected as specified.

Acid phosphatase was derived from pooled human seminal plasma, which was stored at −18 °C. Repeated freezing and thawing with removal of precipitates resulted in a solution in which enzymatic activity was stable for over 10 years. Dilution of the enzyme was performed using buffer with the addition of 0.6 g/l albumin.

In this study sera from patients with elevated catalytic concentration of acid phosphatase were used as well as sera enriched with prostatic acid phosphatase from seminal plasma. Some of the experiments were performed with diluted acid phosphatase from seminal plasma as indicated. No determinations involving inhibition by tartrate were performed within this study.

Reactions were carried out in 0.4 mol/l sodium acetate buffer prepared at 25 °C. The pH/temperature gradient of this buffer is 0.003 pH/ °C. All solutions except buffer were freshly prepared.

Instruments

Reaction kinetics were followed using a Perkin Elmer Double Beam Spectrophotometer 124 with recorder 56 or a PYE Unicam 8800 Spectrophotometer (Philips) with 6 changeable cuvettes. Temperature was controlled to 0.1 °C in a thermostated cuvette holder by a Lauda ultra thermostat.

Procedure

In order to facilitate performance of varying reaction conditions each assay consisted of

1 ml naphthyl phosphate (different concentrations) in sodium acetate buffer (0.4 mol/l, pH 5.2)
1 ml 6.0 mmol/l Fast Red TR + 9 g/l albumin in sodium acetate buffer (0.4 mol/l, pH 5.2)
1 ml sodium acetate buffer (0.4 mol/l, pH 5.2) with other components (e. g. alcohols) added.
0.2 ml serum or diluted acid phosphatase.

The components were mixed without addition of serum, and absorbance was recorded for 10 minutes at 390 nm in 1 cm cuvettes (blank value). Serum was then added, and the reaction was recorded for 20 min at 390 nm. After the lag phase of 5 minutes (5) the mean of the next 10 minutes was used for calculation of ΔA/min.

Enzyme catalytic concentration is derived in U/l = 1176 · ΔA/min using a molar lineic absorbance of the chromophore of $\varepsilon_{390\,nm}$ = 1.36 m²/mol (5). Calculations of kinetic data were made by the use of the kinfit program as described (6).

Results and Discussion

Stability of prostatic acid phosphatase in serum

Enzymatic activity of prostatic acid phosphatase in serum tends to decrease during storage (7, 8). This process is dependent on time, temperature and pH value. We found that inactivation of acid phosphatase and of the enzyme in serum followed first order kinetics at all pH values and temperatures studied. This represents an addition to the existing data on purified prostatic acid phosphatase at single pH values (9, 10). Inactivation of acid phosphatase is plotted in figure 1, showing the log of half life times of the enzyme versus temperature. The data for the enzyme at pH 5.2 and enzyme in serum at pH 6.0 and 7.0 show almost parallel plots, whereas the slope at pH 8.2 is considerably less. This may indicate conformational changes in protein structure near to or slightly above physiological pH values, which facilitate denaturation. The same conclusion may be drawn from *Arrhenius* plots which result in decreasing energies of activation for denaturation (tab. 1) with rising pH. These results might also explain the shorter half life times of acid phosphatase in blood during fever with concomitantly reduced enzyme activity in serum.

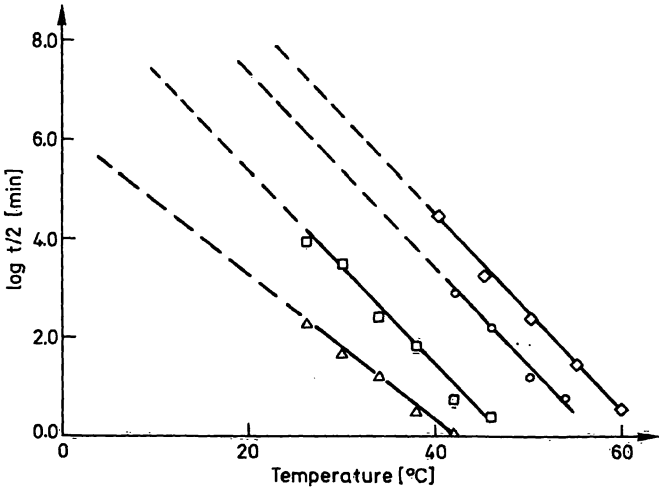


Fig. 1. Half life times of denaturation of prostatic acid phosphatase versus temperature and pH.
◇ — ◇ acid phosphatase from seminal plasma in sodium acetate buffer pH 5.2
○ — ○ acid phosphatase in serum adjusted to pH 6.0
□ — □ acid phosphatase in serum adjusted to pH 7.0
△ — △ acid phosphatase in serum adjusted to pH 8.2

Tab. 1. Energies of activation for denaturation of prostatic acid phosphatase

pH 5.2	380 ± 1 kJ/mol (prostatic enzyme)
pH 6.0	366 ± 2 kJ/mol (in serum)
pH 7.0	348 ± 4 kJ/mol (in serum)
pH 8.2	260 ± 20 kJ/mol (in serum)

Sampling and storage of serum

In clinical chemical determinations the enzymatic activity of prostatic acid phosphatase at the time of assay should not deviate greatly from the enzymatic activity at the time of blood sampling. In this respect the values of figure 1 demonstrate the importance of controlling pH and temperature during storage of serum. In order to guarantee that only sera are assayed in which acid phosphatase is inactivated to less then 10%, storage temperature must be kept below a certain value and pH must be adjusted. In figures 2a and 2b curves are drawn where at least 90% of the original activity is retained with respect to storage time and temperature at a given pH. For a storage time of 5 or 24 hours, 90% activity curves show optimal temperatures that depend on pH (fig. 3).

Since pH of serum in open vessels is raised rather fast (0.5 pH units within the first hour) the time between drawing blood from the patient, clotting and removal of serum is most critical. Thus reduction of clotting time to 15 minutes by using thrombin coated tubes, as suggested by *Theodorsen* (11), clotting in closed vessels, immediate separation of serum, pH adjustment and proper temperature control of the serum sample can not only prevent inactivation of prostatic acid phosphatase considerably but also diminish an increase of phosphatase activity from unintended sources such as platelets and leukocytes.

Dependence of enzymatic activity on pH

A clear maximum of activity is observed at 42 °C at pH 5.6. This maximum levels off below 38 °C, as demonstrated in figure 4. Depending on the substrate,

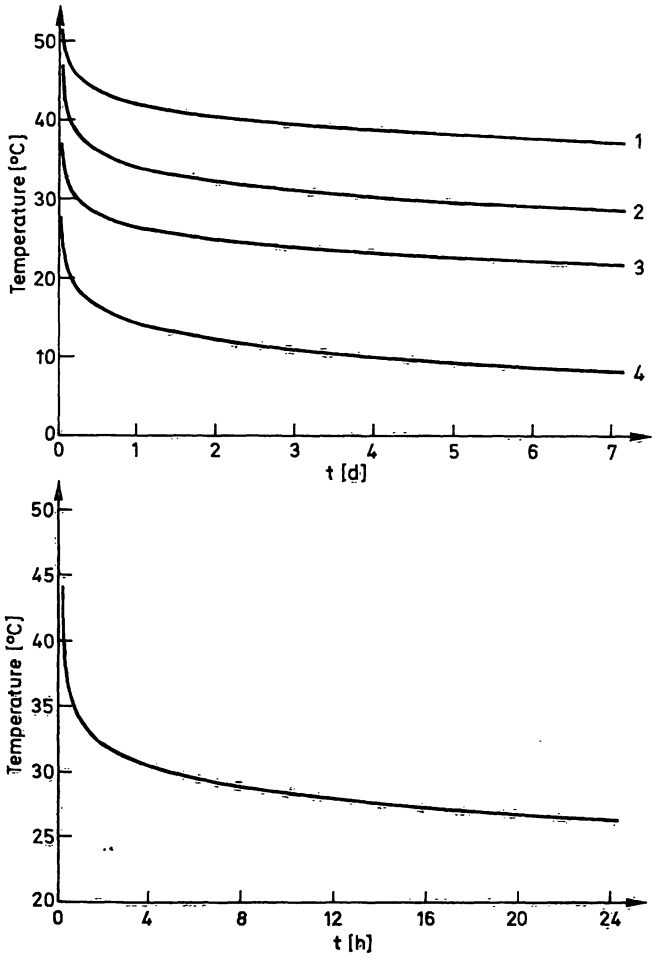


Fig. 2. Permitted time of storage with less than 10% loss of enzymatic activity in dependence on temperature.
a) 1: prostatic acid phosphatase
2: serum pH 6.0
3: serum pH 7.0
4: serum pH 8.2
b) serum at pH 7.0

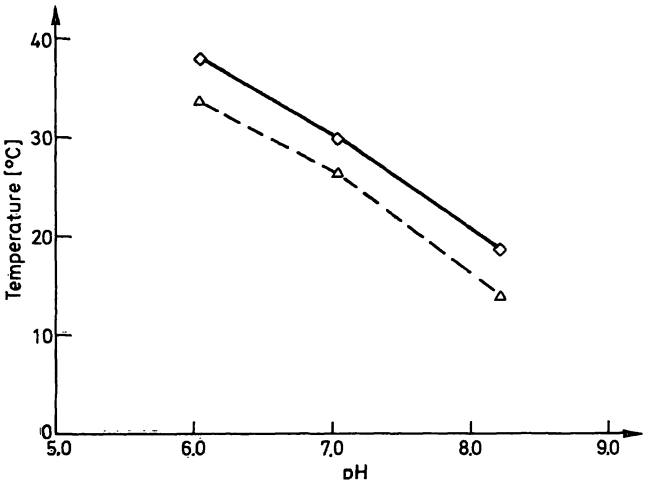


Fig. 3. Influence of temperature and pH on storage. At least 90% of enzymatic activity is retained within 5 hours (upper curve) or within 24 hours (lower curve)

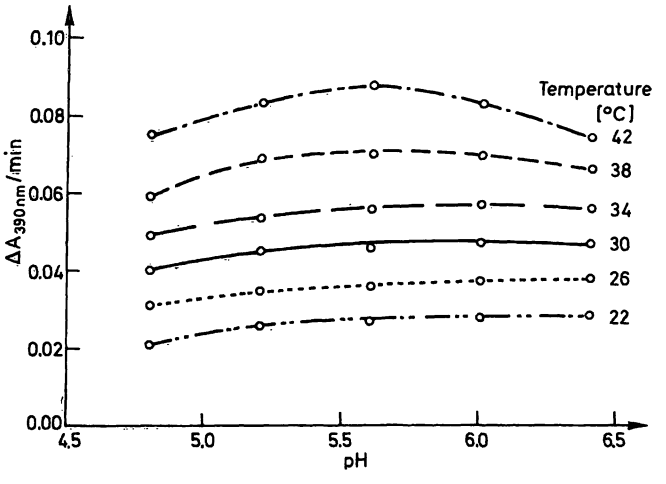


Fig. 4. pH-optimum at different temperatures as indicated.

buffer and the temperature used, pH optima ranging from pH 4.9 (7), 5.2 (12), 5.5 (13), 5.6 (14), 5.7 (15) to pH 6.0 (16, 17) have been reported. But all authors agree that prostatic acid phosphatase shows a broad optimum. The difference of enzymatic activity between pH 5.6 and 5.2 is less than 4%. The indicator reaction with fast red has a shorter lag phase at pH 5.6 than at pH 5.2 (5, 18). But the formation of unspecific chromophores from the blank value as well as from the reaction of proteins with fast red is considerably lower at pH 5.2 (fig. 5) and the influence of bilirubin is minimized at pH 5.2 (19).

Kinetic studies of acid phosphatase revealed that the monoanionic form of the substrate takes part in the kinetically productive reaction step indicated by k_2 in Eq. 1 (20, 21).

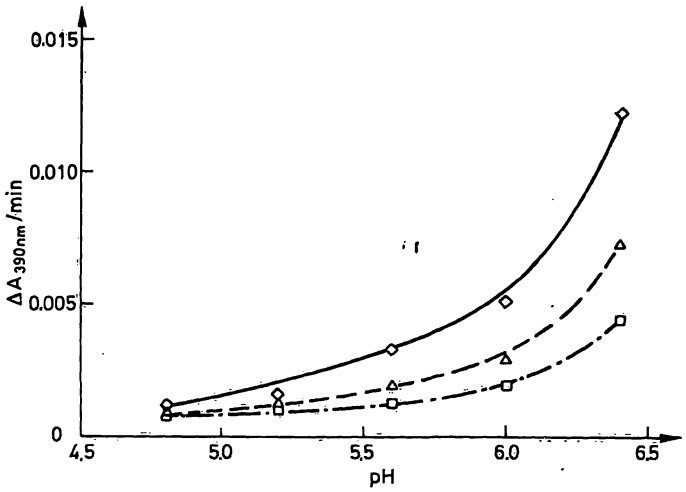
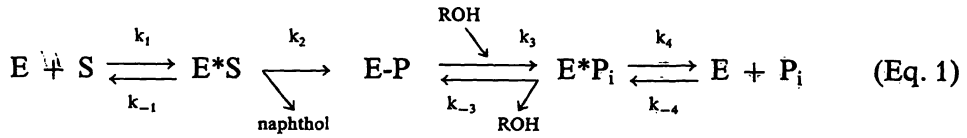


Fig. 5. „Blank values" in serum of healthy blood donors in dependence on pH and temperature.
lower curve: 25 °C
middle curve: 30 °C
upper curve: 37 °C



(Eq. 1)

where

P = product
P_i = inorganic phosphate
R-OH = alcohol or water

In figure 6 $K_{m\,app}$ is drawn in dependence on pH. While $K_{m\,app}$ increases strongly with pH, a slight decrease is observed after correction for substrate ionization using $pK_a = 5.85$ for 1-naphthyl phosphate (22). Substrate saturation of the enzyme thus strongly depends on substrate ionization. Since at pH 5.2 about 82% of the substrate is in the monoionic form and since this value drops to 64% at pH 5.6, and 42% at pH 6.0, it is clear that a lower pH value for the assay of prostatic acid phosphatase is preferable.

From this consideration and the reasons outlined above, a pH of 5.2 is recommended for assay conditions.

Dependence of enzymatic activity on temperature

The maximal velocity of reaction (v_{max}) increases exponentially from 10 to 50 °C. The addition of diols accelerates the enzymatic reaction. But enzyme inactivation occurs at higher temperatures in the presence of 1,5-pentanediol and 1,6-hexanediol at the concentration used (fig. 7).

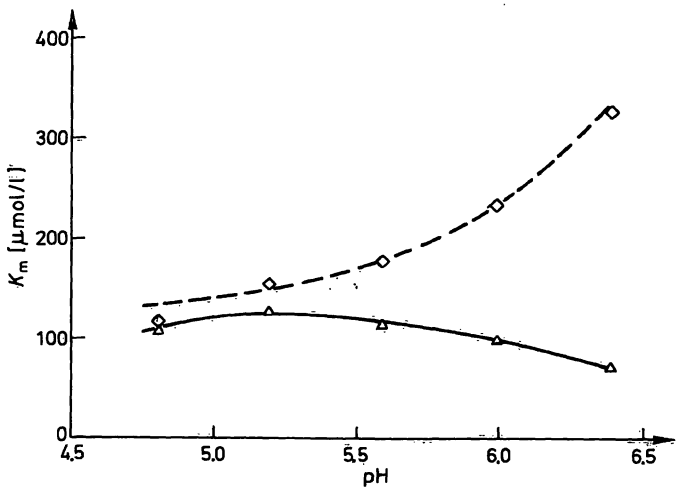


Fig. 6. K_m of prostatic acid phosphatase in dependence on pH
◇ — ◇ $K_{m\,app}$
△ — △ K_m corrected for substrate ionization

The binding constant $K_{m\,app}$ as shown in figure 8 decreases with temperature. Without addition of alcohols it remains relatively constant between 25 and 37 °C. This is similar to the observations with *p*-nitrophenyl phosphate (20). In the presence of diols, however, the temperature dependence is considerable even between 25 and 37 °C. In consequence, in the presence of diols it is difficult to compare results assayed at different temperatures if substrate concentrations are not adjusted to saturation conditions. This is especially valid if the same kit is used at different temperatures.

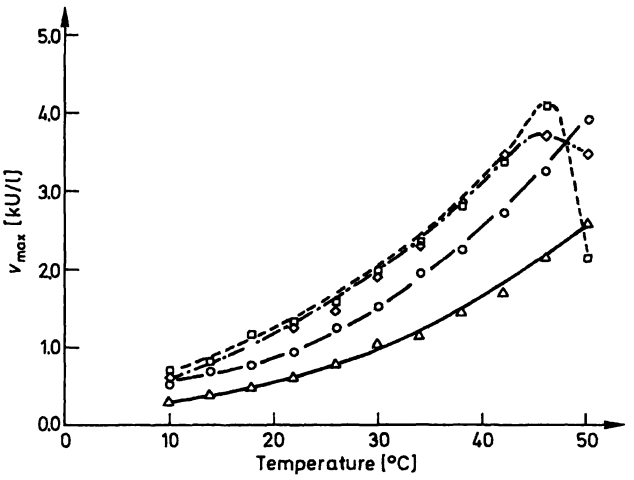


Fig. 7. Dependence of v_{max} on temperature
△—△ enzyme without addition of alcohol
○—○ in presence of 0.25 mol/l 1,4-butanediol
□—□ in presence of 0.25 mol/l 1,5-pentanediol
◇—◇ in presence of 0.25 mol/l 1,6-hexanediol

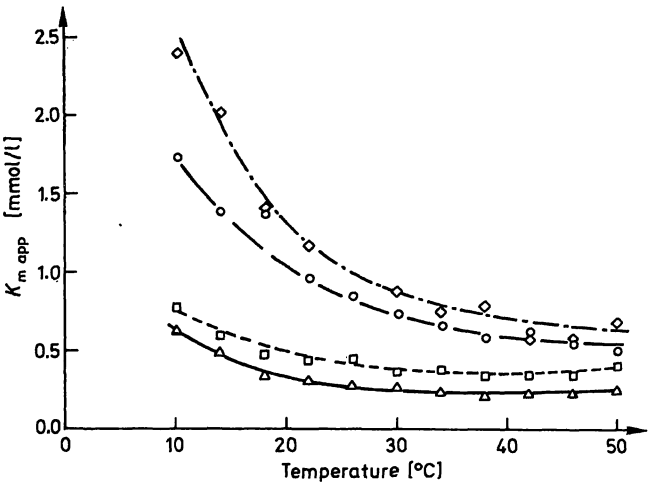


Fig. 8. Dependence of $K_{m\ app}$ on temperature
△—△ enzyme without addition of alcohol
□—□ in presence of 0.25 mol/l 1,4-butanediol
○—○ in presence of 0.25 mol/l 1,5-pentanediol
◇—◇ in presence of 0.25 mol/l 1,6-hexanediol

Energy of activation

It is well known that the *Arrhenius* plots of several enzymes do not show linear regressions between 20 and 40 °C, e. g. glutamate dehydrogenase (23), aminotransferase, lactate dehydrogenase, creatine kinase (24), all being enzymes which need several cofactors (25). But even alkaline phosphatase does not show a linear regression in the *Arrhenius* plot (24). This has been ascribed to the fact that alkaline phosphatase consists of several isoenzymes. Although prostatic acid phosphatase also consists of several isoenzymes a strict linear regression is derived as shown in figure 9 (upper curve). One of the reasons may be that prostatic acid phosphatase mainly consists of isoenzyme 2 according to l. c. (16). But if substrate saturation is not achieved, prostatic acid phosphatase also fails to show linear regression in the *Arrhenius* plot, as demonstrated in figure 9. This might explain deviation from a straight line in multicomponent systems where substrate saturation for all components can hardly be achieved. If assays of acid phosphatase are performed in the presence of diols, deviations from the straight line are likewise observed when using an extended temperature range (fig. 10). Therefore we have calculated the energy of activation between 22 and 42 °C. In the case of acid phosphatase without addition of alcohols no significantly different value is obtained when the energy of activation is calculated using the range between 4 and 50 °C.

The energy of activation has been ascribed to the rate limiting dephosphorylation step in Eq. 1 (20). The value of 10.9 kcal/mol (20) is in good agreement with

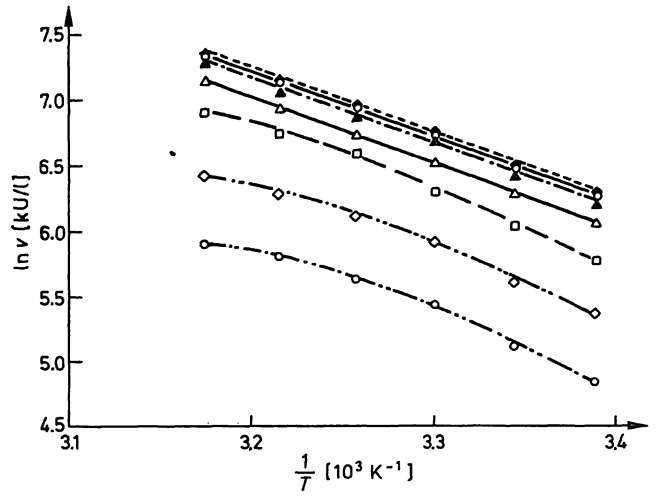


Fig. 9. *Arrhenius* plots of prostatic acid phosphatase-catalysed hydrolysis of 1-naphthyl phosphate at different substrate concentrations.
curves from bottom to top: 0.05, 0.1, 0.25, 0.5, 2.0, 5.0 mmol/l 1-naphthylphosphate and top curve: v_{max}

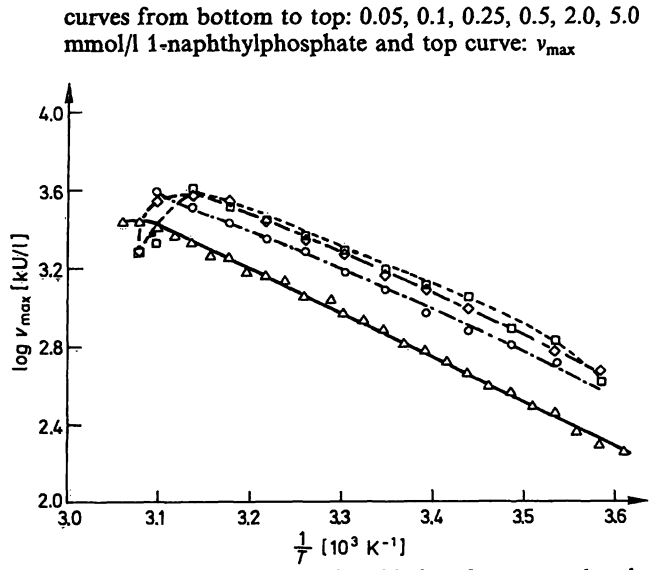


Fig. 10. *Arrhenius* plots of prostatic acid phosphatase-catalysed hydrolysis of 1-naphthyl phosphate at v_{max}
△—△ enzyme without alcohol
○—○ in presence of 0.25 mol/l 1,4-butanediol
□—□ in presence of 0.25 mol/l 1,5-pentanediol
◇—◇ in presence of 0.25 mol/l 1,6-hexanediol

our results. Table 2 shows that alcohols as acceptors of phosphate instead of water decrease the energy of activation. With 1,5-pentanediol a minimum of energy of activation is reached.

The results of figure 8 stress the importance of substrate saturation. But without going into details of reaction kinetics, the possibility of specific changes of the dephosphorylation mechanism cannot be excluded from our data (fig. 10). To avoid unnecessarily high substrate and fast red concentrations with a concomitant increase in production of unspecific chromophores, we suggest that measurements of the catalytic activity of prostatic acid phosphatase should be performed without addition of alcohols, despite the fact that the enzyme is activated by diols.

The straight line of the *Arrhenius* plot (fig. 10) without addition of alcohols enables the application of conversion factors in order to recalculate and compare results from assays performed at different temperatures. These conversion factors are shown in table 3. Since slightly different results are obtained if these conversion factors are applied to sera containing non-prostatic acid phosphatase, care should be taken in using these factors without mentioning the fact of recalculation.

Tab. 2. Energies of activation for prostatic acid phosphatase-catalysed hydrolysis of 1-naphthyl phosphate.

Prostatic enzyme	43.0 kJ/mol
in presence of 0.25 mol/l 1,4 butanediol	39.0 kJ/mol
in presence of 0.25 mol/l 1,5 pentanediol	37.0 kJ/mol
in presence of 0.25 mol/l 1,6 hexanediol	38.6 kJ/mol

Tab. 3. Conversion factors of catalytic activity measured at different temperatures.

	To obtain activity at		
	25 °C	30 °C	37 °C
	multiply by factor		
Assay temperature			
25 °C	–	1.33	1.96
30 °C	0.75	–	1.47
37 °C	0.51	0.68	–

Although it seems feasible from our data to calculate and compare conversion factors for acid phosphatase activity in the absence and presence of alcohols (e. g. pentanediol), we omit these factors in our paper, because they are strongly dependent on the source of acid phosphatase in the serum, and on the alcohol concentration used.

References

1. Aumüller, G., Pohl, C., van Etten, R. L. & Seitz, J. (1981) Virchows Arch. (Cell Pathol.) *35*, 249–262.
2. Hillmann, G. (1971) J. Clin. Chem. Clin. Biochem. *9*, 273–274.
3. Hüting, J., Gundlach, G. & Bleyl, H. (1984) Med. Welt *35*, 1486–1489.
4. Oremek, G., Seiffert, U. B., Heinert, G., Siede, W. H. & Rockenbach, J. (1986) Lab. Med. *10*, 171–175.
5. Gundlach, G. & Mühlhausen, B. (1980) J. Clin. Chem. Clin. Biochem. *18*, 603–610.
6. Knack, I. & Röhm, K. H. (1981) Hoppe-Seyler's Z. Physiol. Chem. *362*, 1119–1130.
7. Doe, R. P., Mellinger, G. T. & Seal, U. S. (1965) Clin. Chem. *11*, 943–950.
8. Josephson, L. & Houle, P. (1980) Clin. Chem. *26*, 1631.
9. London, M., Wigler, P. & Hudson, P. B. (1954) Arch. Biochem. Biophys. *52*, 236–246.
10. Bais, R., Huxtable, A. & Edwards, J. B. (1983) Ann. Clin. Biochem. *20*, 374–380.
11. Theodorsen, L. (1985) Scand. J. Clin. Lab. Invest. *45*, 57–65 (suppl. 179).
12. Shaw, L. M., Brummund, W. & Dorio, R. J. (1977) Am. J. Clin. Pathol. *68*, 57–62.
13. Jacobsson, K. (1960) Scand. J. Clin. Lab. Invest. *12*, 367–380.
14. Warren, R. J. & Moss, D. W. (1977) Clin. Chim. Acta *77*, 179–188.
15. Bais, R. & Edwards, J. B. (1976) Clin. Chem. *22*, 2025–2028.
16. Lam, K. W., Li, O., Li, C. Y. & Yam, L. T. (1973) Clin. Chem. *19*, 483–487.
17. Roy, A. V., Brower, M. E. & Hayden, J. E. (1971) Clin. Chem. *17*, 1093–1102.
18. Escribano, J., Garcia-Carmona, F., Garcia-Canovas, F., Iborra, J. L. & Lozano, J. A. (1984) Biochem. J. *223*, 633–638.
19. Hoffmann, G. E., Hiefinger, R. & Weiss, L. (1985) J. Clin. Chem. Clin. Biochem. *23*, 759–763.
20. van Etten, R. L. & Saini, M. S. (1982) Arch. Biochem. Biophys. *219*, 155–162.
21. Hickey, M. E., Waymack, P. P., van Etten, R. L. (1976) Arch. Biochem. Biophys. *172*, 439–448.
22. Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) Data for Biochemical Research, 2nd Ed., Oxford at the Clarendon Press, pp. 452–453.
23. Jung, K., Egger, E., Neumann, R. & Lüdtke, B. (1974) J. Clin. Chem. Clin. Biochem. *12*, 159–165.
24. Szász, G. (1974) J. Clin. Chem. Clin. Biochem. *12*, 166–170.
25. Jung, K., Lüdtke, B. & Egger, E. (1975) J. Clin. Chem. Clin. Biochem. *13*, 179–181.

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